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13. ABSTRACT (Maximum 200 Words)
Preselection and postselection human mammary epithelial cells (HMECs) differ greatly in replicative potential. In this report, we studied the role of p53 in senescence of preselection and postselection HMECs. Western blot analysis of early (proliferating) and late (senescing) preselection and postselection HMECs showed that postselection HMECs have higher level of p53, which is upregulated during senescence. Postselection HMECs also showed higher expression of p53 target gene p21. On the other hand, there was no increase in p53 level and its target gene p21 in senescent preselection cells. DNA binding assays using p53 binding site of p21 promoter and extracts prepared from early and late passage postselection cells suggest that p53 binding activity increases with senescence in postselection HMECs. We also studied posttranslational modifications of p53 in HMECs using antibodies specific for various phosphorylated and acetylated form of p53. Results suggest there are no major differences in acetylation or phosphorylation of p53 in preselection and postselection HMECs. However, postselection HMECs contained higher level of p53 phosphorylated at Ser-38. Taken together, these results suggest p53 plays an important role in senescence of postselection HMECs, but not in senescence of preselection HMECs

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INTRODUCTION

Most normal human cells undergo replicative senescence after accruing certain number of cell divisions (1). Telomere shortening is believed to be main cause of replicative senescence in human cells (1, 2). p53 tumor suppressor and its target genes such as p21 mediate telomere-shortening signal and invoke permanent growth arrest (3). However, cells also undergo senescence independent of telomere shortening (4). When mammary tissue is explanted in an appropriate tissue culture medium, a heterogeneous cell population emerges. This heterogeneous population proliferates for 3-5 population doublings before a majority of cells undergoes senescence. Regular feeding of these cells (sometimes) give rise to a homogeneous population which is referred to post-selection human mammary epithelial cells (HMECs), while the original heterogeneous mixture is referred to as pre-selection HMECs (5, 6). Senescence in pre-selection cells is also termed as M0 stage (7). During M0 senescence, p16 upregulation and loss of p16 expression in post-selection HMECs has been reported (7, 8). The role of p53 in senescence of pre- and post-selection cells is not well understood.

p53 plays an important role in DNA damage induced apoptosis, G1 arrest, quiescence and senescence associated growth arrest (3, 9). In response to various stimuli associated with apoptosis, quiescence and senescence p53 undergoes a myriad of posttranslational modifications (10, 11). These modifications include acetylation and phosphorylation. All N-terminal serines (Ser 6, Ser 9, Ser 15, Ser 20, Ser 33, Ser 37, Ser 46) and theronines (Thr 18, Thr 55 and Thr 81) in the first 89 residues of human p53 may be phosphorylated or dephosphorylated in response to one or more stress conditions. In addition, Ser 315 and Ser 392 are also phosphorylated, Lys 320, Lys 373, and Lys 382 are acetylated and Lys 386 is sumoylated in response to DNA damage (10, 11). Kinetics of phosphorylation and acetylation depends on the nature of the stimulus. It was recently reported that p53 is acetylated at Lys 382 during ras and PML induced premature senescence (12). In most cases phosphorylation and acetylation of p53 increases its transcription activation potential (10, 11). p53 binding activity and transcription activity increases with senescence in human fibroblasts (9, 13). In human fibroblasts, increased phosphorylation of p53 at Ser 15, Thr 18, Ser 376 and Ser 392 during senescence has been reported (14). Virtually nothing is known about posttranslational modifications of p53 during senescence of pre- and post-selection HMECs. In this report, we studied the role of p53 in senescence of pre- and post-selection HMECs and determined posttranslational modifications associated with p53 during senescence in HMECs.

BODY:

76N pre- and post-selection cells were obtained from Dr. Vimla Band. These cells were cultured in DFCI-1 medium as described (15). Cells were serially passaged in culture until senescence. Senescence was determined using senescence associated beta-galactosidase (SA-β-gal) assay and using ^{3}H-thymdine incorporation assay (% labeled nuclei or %LN) as described (15, 16). Cells were considered early passage when >70% cells incorporated ^{3}H-thymidine and less than 5% cells were SA-β-gal positive. Conversely cells were considered senescence when SA-b-gal index was >70% and %LN were 10-15%. SA-β-gal is a widely used senescence marker used in various cell types including HMECs.

To determine p53 binding activity, gel shift assays were performed as described (9, 17). Briefly, nuclear extracts were prepared from early and senescent post-selection HMECs as described (9, 17). A double strand oligonucleotide (5' GGTCAGGAACATGT CCCAACAT G TTGACC) containing p53 binding site (underlined) present in p21 promoter was end labeled using γ-^{32}P. The labeled oligo was incubated with 2 μg of nuclear extract from early passage and senescent cells for 30 minute at room temperature. The DNA-protein complexes were separated on a 5% native polyacrylamide gel made and run in 0.5% TBE, and autoradiography performed to detect the DNA-protein complexes.

Western-blot analysis was performed as described (14). DO-1 mouse monoclonal antibody that detect total p53 was from Santa Cruz Biotechnology, Santa Cruz, CA. Antibodies to detect acetylated p53 were obtained from Upstate Biotechnology, Lake Placid, NY. A phospho-p53 sampler kit was obtained from Cell Signaling Technology, Beverly, MA.

Research accomplishments

1. p53 DNA binding activity at various stages of cellular senescence in HMECs:

Post-selection HMECs were serially passaged in culture and frozen at different passages. SA-β-gal index and %LN of HMECs were determined at each passage. At passage 17, these HMECs appear to be senescent as determined by %LN (<15%) and SA-β-gal index (>70%). Similarly, cells at passage 11 were considered early passage by determining %LN (>80%) and SA-β-gal index (<5%). Nuclear extracts were prepared from cells at passage 11, passage 15 and passage 17, and p53 DNA binding assay was performed as described above. The results (Fig. 1) suggest that p53-binding activity modestly increases in senescent HMECs compared to early passage cells. Binding specificity of p53 was confirmed using competition with wild type and mutant (containing mutant p53 binding site) oligos (Fig. 1).

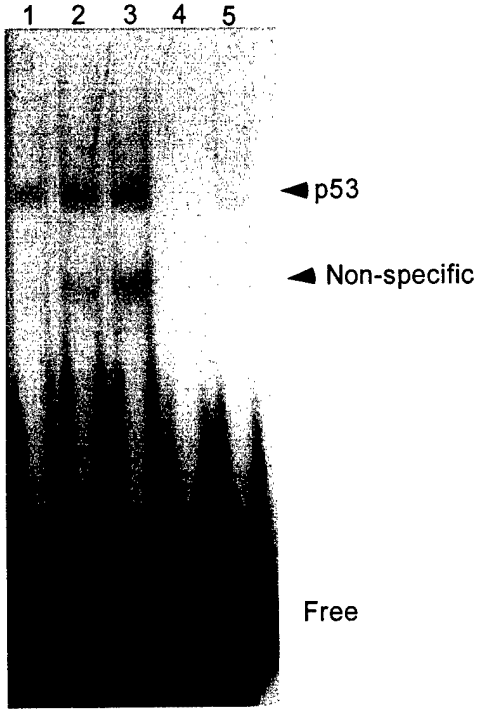

Figure 1: p53 DNA binding activity increases with senescence in post-selection HMECs. Lane 1, 2 and 3 represent nuclear extract from HMECs at passage 11 (early passage), 15 (mid passage) and 17 (senescent) respectively. Lane 4 is competition using 100-fold excess wild type

oligo, while lane 5 is competition using 100-fold excess mutant oligo (containing mutant p53 binding site). In competition assays, nuclear extract from passage 11 was used. Arrows indicate p53 specific and non-specific band (as indicated). Free is the labeled probe that did not bind p53.

2. Posttranslational modifications of p53 during senescence in HMECs:

Posttranslational modifications of p53 during senescence in HMECs was studied by western blot analysis using antibodies that detects total p53 or phosphorylated and acetylated forms of p53. Total cell extract was prepared from early passage and senescent pre-selection and post-selection HMECs. Equal amount of extract (40 μg) was run on 5-15% gradient polyacrylamide gel, transferred to PDVF membrane and probed with various antibodies as described (13). The results (Fig. 2) suggest that p53 is downregulated in senescent pre-selection cells but is upregulated in senescent post-selection cells. Furthermore expression of p21, a p53 target gene, which is a CDK inhibitor, and known to be upregulated during senescence in fibroblasts correlates well with p53 level. Senescent post-selection but not pre-selection HMECs contained increased amount of p21 protein.

As expected post-selection cells contained no p16 and as reported (7, 8), p16 was upregulated in senescent pre-selection HMECs. When probed with antibodies specific for acetylated p53 (Lys 320, and Lys-373, Lys-382), results show no significant difference in steady state level of acetylated p53 (Fig. 2). Although, there appears to be modest increase in acetylated p53 in senescent post-selection HMECs compared to early post-selection HMECs when probed with antibody specific for Lys-320. Western blot analysis using antibody specific for Ser-15 and Ser-37 showed that there p53 phosphorylated at Ser-37 is increased in post-selection HMECs, but its level do not increase with senescence. On the other hand, Ser-15 phosphorylation remained mostly unchanged in pre-and post-selection cells but senescent post-selection HMECs exhibited slight increase when compared to early passage cells. (Fig. 2).

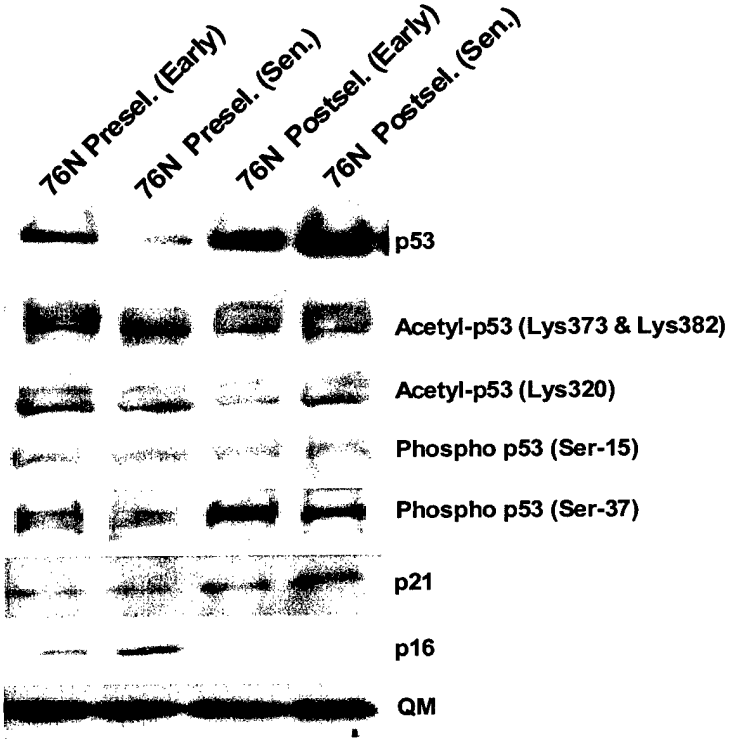

Figure 2: Steady state level of p53 and its transcription activity increases with senescence in post-selection but not in pre-selection HMECs. Western-blot analysis using indicated

antibodies to determine the expression of various proteins (p53, p16, p21 and QM), and posttranslational modifications of p53 (acetyl and phospho p53) was performed as described in the text. QM is a loading control that does not change with senescence in human fibroblasts (18).

KEY RESEARCH ACCOMPLISHMENTS:

During past one year, I have learned how to culture pre- and post-selection HMECs, and determine senescence in these cells. I have also optimized p53 DNA binding and chromatin-immunoprecipitate linked PCR (ChIP) assay. The key research accomplishments during past year are following:

- p53 DNA binding activity increases with senescence in post-selection HMECs.
- p53 level and its transcription activity as determined by examining the level of its target gene p21 increases with senescence in post- but not pre-selection HMECs.
- There are no significant posttranslational changes in p53 during senescence in HMECs as determined by a limited set of antibodies.

REPORTABLE OUTCOMES: None

CONCLUSIONS:

p53 an important mediator of cellular senescence, which plays a role in telomere length dependent senescence. Gradual telomere shortening is thought to provoke a DNA damage checkpoint mediated by p53, which results in permanent growth arrest. Most tumor cells have lost this ability to undergo senescence and cycle even when telomere lengths critically short. In this report, we have presented evidence that p53 may play an important role in senescence of post-selection cells but not pre-selection cells.

In the first year of the grant, we proposed to study the DNA binding activity, its expression level and posttranslational modifications during senescence in HMECs. We have completed the proposed studies. However, we have not found any significant differences in posttranslational modifications using limited number of antibodies that we used. Hence, we plan to continue studying posttranslational modification studies using additional antibodies specific to phospho- and acetylated form of p53.

P53-abrogating mechanisms can extend the replicative life spans of cells. Under such circumstances, cells keep dividing and ignore the stop signal, resulting in continued telomere shortening and significant extension of replicative life span. Ultimately, when telomeres become critically short and genomic abnormalities arise due to chromosome fusion's, cells undergo crisis, whereby most cells in culture undergo cell death. We have recently started using p53 RNAi approach to study the role of p53 in senescence. We plan to abrogate p53 using RNAi approach in pre- and post-selection HMECs. We expect stable expression of p53 RNAi using a retrovirus approach (19) to overcome senescence in post-selection HMECs but not in pre-selection HMECs. p53 RNAi studies should complement results presented here and give us

unambiguous results about the role of p53 in mammary epithelial cell senescence. Depending on the outcome of p53 RNAi studies, we will perform p53 ChIP analysis in pre- and post-selection HMECs as previously proposed in the grant application.

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